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EFFECT OF DIFFERENT ENVIRONMENTAL CONDITIONS ON QUALITY OF DNA EXTRACTED FROM HUMAN TEETH FOR GENDER DETERMINATION

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ABSTRACT
Use of morphological characteristics in human identification maybe difficult in conditions where* human *remains are grossly fragmented, decomposed and/or mutilated, or when only a tooth is available. The study aims to ascertain and compare the quantity of DNA from teeth exposed to different environmental conditions using organic and silica column DNA extraction methods and determine their gender using amelogenin gene primer. Human teeth were used and divided into four major groups based on different exposure types (buried in soil, storage in Lagoon water, normal environmental condition by placing them on the floor at the botanical garden, and exposure to temperature of 210^{o}C for 15mins). The powdered dentin-cementum complex was used for DNA extraction, quantification and purity assessments. Amplification was done using amelogenin gene primers. Mean DNA concentration and purity were 187.59±26.25ng/µl and 0.97±0.07 respectively for silica column method, and, 138.98±35.71ng/µl and 0.91±0.11 respectively for organic extraction. With organic extraction, the least and highest mean DNA concentration are from teeth immersed in Lagoon water (74.86±61.19ng/µl) and teeth exposed to temperature of 210^{o}C for 15mins (385.14±106.39ng/µl). In the Silica column method, teeth heated at 210^{o}C for 15mins showed the lowest mean DNA concentration (165.57±33.82ng/µl) while teeth exposed to "normal environmental conditions", gave the highest DNA concentration (218.46±45.03ng/µl). Positive and negative male identification were 30.8% and 69.2% respectively, while female identification was 100% positive irrespective of the environmental exposure. The dentine-cementum is a valuable DNA source for forensic investigations. Male identification using amelogenin gene, should still be investigated in the Nigerian context.
Keywords: Sex determination; Dentin-cementum complex; Amelogenin gene; Silica column method; Organic extraction.

INTRODUCTION

Forensic Odontology is an important aspect of forensic science and has played a major role in human identification. It uses dental and orofacial investigations in providing solutions to legal and criminal issues (Vemuri *et al.,* 2012: Dinakaran *et al.,* 2015). Additionally, it helps in human identification, in mass disasters, bioterrorism, identify a missing person and highly degraded bodies (Nayar *et al.,* 2017; Shah, 2019). Dental analysis is a quick and low cost procedure, though it also has the advantage of relying in the integrity of the teeth – which are the most durable part of the human body (Belotti *et al*., 2015).

DNA often persists in teeth much longer than in the soft tissues of the body, because the rigid structure of the teeth protects it from degradation (Latham and Miller, 2018). This feature enables its natural ability to survive harsh conditions, a property that forensic scientists and researchers, are maximizing, to aid human identification (Vemuri et al., 2012: Dinakaran et al., 2015).

Due to the relatively high degree of physical and chemical resistance of the dental structure to elements such as fire, flames, heat, and explosions, the teeth play an important role in identification and criminology (Morgan *et al*., 2006). Dentine cementum complex is a good source of DNA for personal identification, especially when the specimen is highly decomposed, burnt or degraded since they are highly calcified structures.

In forensic studies, data collection is an integral part of the identification to enable credible investigations. Hence, information regarding the sex is the first step in biological profiling, positive human identification in cases of, disasters, crime investigations, anthropology studies and ethnic studies (Ramakrishnan, *et al.,* 2015). Determination of the sex randomly from a single tooth is extremely difficult (Vemuri *et al.,* 2012). But with the proliferation of molecular technology, forensic analysts have turned to its use, as deoxyribonucleic acid (DNA) can be isolated and recovered from a highly degraded sample where the traditional, morphological techniques, may no longer be useful (Kalistu and Dogalli, 2016). Recent approaches to DNA profiling supports the use of Amelogenin markers, which can be used for gender determination (Iwamura *et al.,* 2016).

Several studies have been carried out using human teeth samples for DNA quantification and sex identification, and have tried to mimic various forensic conditions such as exposure to sea or saline water, burial under the soil, high temperatures, under varying durations (Zapico and Uberlaker, 2013; Dutta et al., 2017; Nayar et al., 2017; Chowdury et al., 2018).

Environmental factors such as weather conditions, burial, exposure to Lagoon water and heat can affect the biological degradation process of DNA in hard tissues (Latham and Madonna, 2013; Latham and Miller, 2018) hereby affecting human identification parameters. Other studies have either used single DNA extraction methods like DNA extraction performed using Chelex-100 (Reddy *et al*., 2011), silica-based method (Zapico and Ubelaker, 2013; Kholief *et al*., 2017), phenol-chloroform method (Chowdhury *et al*., 2018; Dutta *et al*., 2017; Kumar and Aswath 2016).

However, what has not been established is the comparison of the two most common DNA extraction methods- the solution-based or chemical-based DNA extraction method e.g. Phenol-chloroform method and the Solid-phase DNA extraction method e.g. Silica column-based DNA extraction method, in the extraction of possible degraded DNA of teeth exposed to the different forensic insults carried out in this study. This is to ascertain the DNA extraction method that, is more efficient for extraction of DNA from the tooth sample, isolate high quality and high purity DNA and also produce sufficient amount of DNA for use in downstream processes. Hence this study aims to quantify nuclear DNA of teeth exposed to different environmental conditions, determine gender using *amelogenin* gene, and compares two DNA extraction methods, organic and silica column methods.

MATERIALS AND METHODS

Ethical approval

The aim of the research was explained to the participants and they willingly and fully consented to participate in the research. Prior to sample collection, the participants completed and returned the short questionnaires in surveys. Informed written and oral consent was obtained. The study was approved by the Health Research Ethic Committee of the College of Medicine, University of Lagos, with approval number CMUL/HREC/0606/19.

Experimental design

The teeth were obtained in collaboration with the Oral and maxillofacial Department Dental Clinic at Lagos University Teaching Hospital (LUTH). This study involved patients undergoing periodontal, orthodontic, and or prosthetic tooth extractions. The date of birth, date of extraction, gender, and the reason for extraction were provided by the health personnel and the patients and the data collected was recorded for each tooth.

Sample preparation and purification

After extraction, each tooth was rinsed with normal saline to remove blood deposits and salivary coating. The teeth were decontaminated using sequential washes of 5% hypochlorite and 96% ethanol as described by Kemp and Smith, (2005). Each specimen was subsequently labelled and immediately exposed to any of the four environmental conditions.

Environmental conditions and timeline

The cleaned teeth were air dried, grouped, labelled and exposed to different environmental conditions. Methodology for grouping the teeth under different environmental conditions, by Chowdhury et al., (2018), was modified to accommodate varying durations of exposure.

Group I was exposed to Normal environmental conditions by placing them on the floor at the botanical garden, exposed to sunlight for the period of 1-10 days (subgroup N1), 11-20 days (subgroup N2) and 21-30 days (subgroup N3).

Group II was immersed in Lagoon water. Each tooth was individually placed in bag of strong rubber thread worked into an open meshed fabric and immersed at the lagoon in University of Lagos, Nigeria for 1-10 days (subgroup LW1), 11-20 days (subgroup LW2) and 21-30 days (subgroup LW3).

Group III was buried 30 cm deep into the soil (sandy) for 1-10 days (subgroup B1), 11-20 days (subgroup B2) and 21-30 days (subgroup B3).

Group IV was exposed to a temperature of 210°C for 15mins (subgroup H) using a temperature controlled oven.

After the experimental period, the teeth were sectioned horizontally. The crown and enamel of the tooth were removed using a dental bur. The dentine cementum complex (root of the teeth), was pulverized using a metal mortar and pestle into powder and collected into a sterile tube for further analysis. Double distilled sterile water was used as negative control (Chowdhury *et al*., 2018).

DNA Extraction

Two methods of DNA extraction were used-: silica column and organic extraction method. The silica column-based method was performed using the Zymo Quick DNA mini prep plus kit for blood and tissue, with Catalogue no D4068 and D4069 (Zymo Research, California USA). The manufacturer's instruction was followed. The organic extraction method (Phenol-Chloroform) was performed using Kumar and Aswath, (2016), method. The DNA was eluted with 50µl of DNA elution buffer and stored at -70°C pending DNA quality and quantity check using the DNA spectrophotometer, and agarose gel electrophoresis.

DNA Quantification and Purity

The eluted DNA (10µl) was added to 5µl of DNA loading dye, and was placed in the lanes of the already cast 2% agarose gel mixed with ethidium bromide. Electrophoresis was done using 5X TBE buffer, at 65 V for 30 min. The gel was viewed using a UV transilluminator (MacroVue UVis-20) and accessed for purity (Zapico and Uberlaker, 2013).

A spectrophotometric check was performed to access DNA quality and quantity. The NanoDrop 1000 spectrophotometer (ND-1000 Spectrophotometer; NanoDrop Technologies; Thermo Fisher Scientific, Inc., Waltham, MA, USA) was calibrated and appropriate wavelengths of 260nm, 280nm, and 230nm were chosen. The spectrophotometer was blanked using elution buffer and nuclease-free water respectively. DNA was quantified by measuring absorbance at 260nm and 280nm (A260/A280) and 260nm and 230nm (A260/A230). The absorbance quotient (O.D. 260/O.D. 280) value of 1.7-2.0 was considered to be purified DNA. A ratio of <1.8 is indicative of protein contamination and a ratio of >2.0 indicates RNA contamination (Khare *et al.,* 2014)

DNA amplification and Polymerase Chain Reaction (PCR).

Amplification and gender determination were possible even when DNA was undetectable using the spectrophotometer. The DNA extracted from teeth samples with the silica column and organic extraction methods were used. A standard polymerase chain reaction (PCR) technique, was used to amplify DNA and the AMEL gene complementary primers used were

D3S1358F Forward 5'-TATGACCCCCACTGCAGT-3',

D3S1358R Reverse 5'-ATGAAATCAACAGAGGCTTGC-3' (Sullivan *et al.,* 1993).

This was carried out using EDVO cycler in 20µl reactions that contained 4.0µl of PCR Buffer 10×, magnesium chloride ($MgCl_2$), 1.5mM, 1mM of deoxynucleotide triphosphates (dNTPs), 0.5 unit of Biolase enzyme (DNA Taq polymerase) from Bioline and 0.4µl of forward and reverse primers, 13.2µl water. The thermocycling conditions were done as follows: one cycle at 95°C for 3 min, 40 cycles under the conditions of 95°C for 30 sec, 60°C (primers annealing) for 45 sec and 72°C for 45 sec. The final step was one cycle at 72°C for 10 mins (Zapico and Uberlaker, 2013).

Visualization of amplified PCR products was done via gel electrophoresis in a 2% agarose gel containing ethidium bromide conducted for 1 hour at 100 V. After electrophoresis, amplicon bands were visualized under UV light. The presence of one band indicates XX (female), while the presence of two bands indicates XY (male) (Zapico and Uberlaker, 2013).

STATISTICAL ANALYSIS

This was done using SPSS software 21 (SPSS Inc., Chicago, IL). All the data were represented as the mean± the standard error of mean (SEM). Independent T-test, Pearson Correlation, chi square, ANOVA was used to find the differences between groups and P values ≤0.05 were considered significant.

RESULTS

Table 1 shows that silica column method of DNA extraction, gave more DNA yield compared to the organic method of extraction in all the groups except in the group heated to 210^{o}C for 15mins where DNA concentration and purity were higher using organic method of extraction. No DNA was extracted from the teeth in group N3 after three trials using the organic extraction method.

Table 1: DNA concentrations and purity from different environment of exposure (groups).

Groups	Organic Extraction Method			Silica Column Method		
	Conc (ng/µl)	**260/280nm**	**260/230nm**	**Conc (ng/µl)**	**260/280nm**	**260/230nm**
B1	121.33±87.78	1.39±0.09	0.99±0.1	272±76.94	1.12±0.02	0.66±0.06
B2	97±2	1.16±0.33	0.51±0.18	203±3	1.15±01	0.6±0.1
B3	74.8±41.6	1.26±0.42	0.52±0.14	80±30.85	0.77±0.25	0.29±0.13
H	385.14 ±106.4	1.15±0.07	0.77±0.06	165.57±33.82	0.98±0.16	0.49±0.14
N1	150±136.72	0.81±0.42	0.6±0.3	252±47	1.20±0.09	0.50±0.16
N2	120.33±105.1	0.88±0.28	0.46±0.16	219.67±75.27	0.93±0.19	0.47 ±0.13
N3	-	-	-	193.67±86.19	1.11±0.01	0.78±0.03
LW1	152.83±142.86	0.95±0.36	0.61±0.14	255.4±112.14	1.16±0.05	0.33±0.12
LW2	40±20.99	0.65 ±0.35	0.26±0.26	203.17±119.84	0.77±0.24	0.4±0.16
LW3	2.2±2.2	0.39±0.39	0.13±0.13	51±25.78	0.76±0.38	0.35±0.25

KEY:

B1 = buried (1-10 days)
B2 = buried (11–20 days)
B3 = buried (21–30 days)
H = heated at 210^{o}C (15mins)
N1 =Normal environment (1-10 days)
N2 = Normal environment (11–20 days)
N3 = Normal environment (21–30 days)
LW1 = Lagoon water (1-10 days)
LW2 = Lagoon water (11–20 days)
LW3 = Lagoon water (21–30 days)

Table 2 shows the DNA concentration of the organic form of extraction and the silica column extraction method gotten from the different environments. Teeth samples immersed in Lagoon water gave the least mean DNA concentration of 74.86±61.19ng/µl, followed by teeth that were buried, 97.71±45.37ng/µl, with the highest concentration being from teeth exposed to temp at 210^{o}C (385.14±106.39ng/µl). While in Silica column method of extraction, teeth exposed to heat at 210^{o}C for 15mins (165.57±33.82ng/µl) showed the lowest mean DNA concentration compared to other groups. DNA Purity at 260/230 nm and 260/280 nm wavelength for both organic and silica column methods of DNA extraction were decreased in teeth immersed in lagoon.

Table 1: Comparison of DNA Concentration with Environment of Exposure (ANOVA)

	Organic Extraction Method			Silica Column Method		
Environment	**conc(ng/µl)**	**260/280nm**	**260/230nm**	**conc(ng/µl)**	**260/280nm**	**260/230nm**
Buried (B)	97.71±45.37	1.27±0.24	0.55±0.1	170.25±43.4	0.96±0.13	0.45±0.09
Heated at 210^{o}C for 15mins (H)	385.14±106.39	1.15±0.07	0.77±0.06	165.57±33.82	0.98±0.16	0.49±0.14
Normal environment (N)	106.55±65.57	0.7±0.21	0.42±0.13	218.46±45.03	1.04±0.11	0.53±0.09
Lagoon (L)	74.86±61.19	0.69±0.22	0.36±0.1	189.21±64.91	0.91±0.13	0.37±0.09

In Table 3, as the time of exposure in all the groups increased, the DNA concentration using organic extraction decreased. DNA purity though low, remained almost stable. For all the groups, DNA concentration was higher using silica column method compared to the organic method except for the heated group.

Table 3: Time of Exposure versus DNA conc and purity

Expose time in all the groups	Organic Extraction Method			Silica Column Method		
	conc (ng/µl)	260/280nm	260/230nm	conc (ng/µl)	260/280nm	260/230nm
1–10 days	117.5±69.48	0.83±0.23	0.51±0.18	247.19 ±47.78	1±0.17	0.46±0.1
11–20 days	95.29±56.26	0.81±0.2	0.37±0.09	157.36±53.97	1.07±0.07	0.47±0.07
21–30 days	69.19±41.1	0.95±0.25	0.48±0.1	133.86±44.9	0.82±0.14	0.41±0.1
15 mins (210^{o}C)	385.14±106.39	1.15±0.07	0.77±0.06	165.57±33.82	0.98±0.16	0.49±0.14

As shown in Table 4, there was no significant difference between the type of tooth and DNA quantity, but the premolars gave the highest concentration in the organic method of extraction while the lower molars gave the highest concentration in silica column method. The canine gave the lowest concentration in both methods.

Table 4: Comparing the DNA concentration from different types of tooth using the two extraction methods

Method of extraction	Type of Teeth	DNA conc (ng/µl) Mean±SEM	F ratio	Sig
Organic Extraction	Upper Molar	47.64±30.40	2.38	0.07
	Lower Molar	173.92±74.61		
	Canine	66.86±57.57		
	Incisors	133.25±138.30		
	Premolar	364.33±133.42		
Silica column	Upper Molar	218.21±51.07	1.32	0.28
	Lower Molar	243.15±58.93		
	Canine	74.86±29.50		
	Incisors	146.75±66.16		
	Premolar	154.50±37.81		

Significant at $P \leq 0.05$

In Table 5, the males had a higher concentration of DNA compared to female despite that more females (30) were analyzed compared to males (14). DNA purity in males were also slightly higher, in both organic and silica column methods of extraction. In PCR identified sex, the females had a higher concentration of DNA and purity compared to the males.

Table 5: Comparison between DNA concentration and gender

	Sex	Organic Extraction Method			Silica Column Method		
		conc (ng/µl)	260/280nm	260/230nm	conc(ng/µl)	260/280nm	260/230nm
Sex	**F**	114.65±36.11	0.87±0.12	0.51±0.07	176.26± 8.52	0.96±0.08	0.45±0.06
	M	197±85.42	1.02±0.26	0.46±0.11	214.62± 58.5	0.97±0.12	0.46±0.1
PCR marker assisted sex identification (PMASI)	**F**	145.73±38.78	0.94±0.12	0.5±0.06	l94.7±28.2	0.98±0.07	0.45±0.05
	M	71.5±62.64	0.63±0.37	0.42±0.18	l16.5±58.4	0.86±0.29	0.44±0.2

In Table 6, positive male identification was 30.8%, while negative male identification was 69.2%. This means that 30.8% of the males were identified correctly as males while 69.2% of males were wrongly identified as females. For the females, 100% of them were positively identified as females. These results are highly significant at P≤0.01.

Table 6: Polymerase Chain Reaction amplification and Identification of AMELX and AMEL Y

		Male	Female	Chi Square (χ)	Sig
PCR marker assisted sex identification (PMASI)	**Male**	30.8%	0.0%	10.178	**0.001**
	Female	69.2%	100.0%		
Total		100.0%	100.0%		

In Table 7, DNA concentration from both extraction methods, are significantly correlated to each other at 0.05 level. DNA concentration from both the silica extraction method, is also significantly correlated with its purity at 260/230nm and 260/280nm at 0.01 level, which implies that the higher the DNA purity at 260/280nm, the higher the purity at 260/230nm. The Table also shows that there was significant correlation between time of exposure, and DNA concentration at 0.05 level. There is a positive correlation between groups (environment of exposure) and time of exposure, at the 0.01 level.

Table 7: Pearson's Correlation between Group, Gender, Organic and Silica Column Extraction Methods, PCR marker assisted sex identification and Time of exposure

				Organic Extraction Method			Silica Column Method				
		Group	**Gender**	**Conc. (ng/µl)**	**260/280nm**	**260/230nm**	**Conc. .(ng/µl)**	**260/280nm**	**260/230nm**	**PCR marker assisted sex identification (PMASI)**	**Time of exposure**
Group	Pea Corr	1	0.02	0.27	0.29	0.29	-0.11	-0.08	-0.05	-0.18	0.71**
	Sig.		0.89	0.08	0.06	0.06	0.48	0.62	0.77	0.24	0.00
Gender	Pea Corr	0.02	1	-0.16	-0.09	0.06	-0.10	-0.01	-0.01	-0.21	0.01
	Sig.	0.89		0.3	0.55	0.69	0.51	0.94	0.93	0.18	0.94
Organic Extraction conc. (ng/µl)	Pea Corr	0.27	-0.160	1	0.179	0.497**	0.318*	0.207	0.270	0.091	0.356*
	Sig.	0.08	0.3		0.25	0.001	0.04	0.18	0.08	0.56	0.02
Organic Extraction 260/280nm	Pea Corr	0.29	-0.09	0.18	1	0.68**	-0.09	0.15	0.21	0.12	0.15
	Sig.	0.06	0.55	0.25		0.00	0.55	0.34	0.17	0.43	0.3400
Organic Extraction 260/230nm	Pea Corr	0.29	0.06	0.5**	0.68**	1	0.03	0.28	0.33*	0.07	0.24
	Sig.	0.06	0.69	0.001	0.0		0.83	0.06	0.03	0.67	0.13
Silica Column conc. (ng/µl)	Pea Corr	-0.11	-0.1	0.32*	-0.09	0.03	1	0.47**	0.5**	0.13	-0.05
	Sig.	0.48	0.51	0.04	0.55	0.83		0.001	0.001	0.4	0.75
Silica Column 260/280nm	Pea Corr	-0.08	-0.01	0.21	0.15	0.28	0.47**	1	0.58**	0.08	-0.12
	Sig.	0.62	0.94	0.18	0.34	0.06	0.001		0.0	0.6	0.45
Silica Column 260/230nm	Pea Corr	-0.05	-0.01	0.27	0.21	0.33*	0.5**	0.58**	1	0.01	-0.01
	Sig.	0.77	0.93	0.08	0.17	0.03	0.001	0.0		0.93	0.96
PCR marker assisted sex identification (PMASI)	Pea Corr	-0.18	-0.21	0.09	0.12	0.07	0.13	0.08	0.01	1	-0.26
	Sig.	0.24	0.18	0.56	0.43	0.67	0.4	0.6	0.93		0.09
Time of exposure	Pea Corr	0.71**	0.01	0.36*	0.15	0.24	-0.05	-0.12	-0.01	-0.26	1
	Sig.	0.0	0.94	0.02	0.34	0.13	0.75	0.45	0.96	0.09	

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed), Pea Corr = Pearson Correlation, Sig = Sig. (2 – tailed)

Table 8, shows that the DNA concentration from both the organic method of extraction and silica column method are significantly correlated with DNA purity at 260/230nm and 260/280nm at level 0.01.

DNA concentration from the organic method of extraction is significantly correlated with the groups of exposure at level 0.01 and DNA purity at 260/230nm from silica column method of extraction at level 0.05. There is also a positive correlation between groups (environment of exposure) and time of exposure at the 0.01 level.

Table 8: Nonparametric Correlations (Kendall's tau_b Correlations) between group, gender, organic and silica column extraction methods, PCR marker assisted sex identification and time of exposure

				Organic Extraction Method			Silica Column Method				
		Group	**Gender**	**Conc. (ng/µl)**	**260/280 nm**	**260/230 nm**	**Conc. (ng/µl)**	**260/280 nm**	**260/230 nm**	**PCR marker assisted sex identification**	**Time of exposure**
Group	Cor Co	1.00	0.01	0.30**	0.16	0.2	-0.07	0.05	-0.02	-0.17	0.66**
	Sig.	.	0.93	0.003	0.17	0.07	0.51	0.68	0.845	0.21	0.0
Gender	Cor Co	0.012	1.00	-0.05	-0.03	0.07	-.075	0.05	-0.01	-0.21	0.02
	Sig.	0.93	.	0.72	0.84	0.59	0.55	0.72	0.95	0.18	0.91
Organic Extraction conc. (ng/µl)	Cor Co	0.33**	-0.05	1.00	0.28*	0.55**	0.18	0.04	0.26*	0.04	0.23
	Sig.	0.003	0.72	.	0.01	0.00	0.09	0.7	0.02	0.74	0.05
Organic Extraction 260/280nm	Cor Co	0.16	-0.03	0.28*	1.00	0.42**	-0.01	0.001	0.16	0.09	0.05
	Sig.	0.17	0.84	0.01	.	0.0	0.92	0.99	0.16	0.49	0.66
Organic Extraction 260/230nm	Cor Co	0.2	0.07	0.55**	0.42**	1.0	0.14	0.17	0.25*	0.05	0.14
	Sig.	0.07	0.59	0.0	0.0	.	0.19	0.13	0.03	0.68	0.26
Silica Column conc. (ng/µl)	Cor Co	-0.07	-0.08	0.18	-0.01	0.14	1.00	0.23*	0.48**	0.11	-0.05
	Sig.	0.51	0.55	0.09	0.92	0.19	.	0.04	0.0	0.37	0.69
Silica Column 260/280nm	Cor Co	0.05	0.05	0.04	0.001	0.17	0.23*	1.0	0.16	0.08	-0.05
	Sig.	0.68	0.72	0.7	0.99	0.13	0.04	.	0.16	0.52	0.68
Silica Column 260/230nm	Cor Co	-0.02	-0.01	0.26*	0.16	0.25*	0.48**	0.16	1.0	0.03	-0.01
	Sig.	0.85	0.95	0.02	0.16	0.03	0.0	0.16	.	0.82	0.94
PCR marker assisted sex identification	Cor Co	-0.17	-0.21	0.04	0.09	0.05	0.11	0.08	0.03	1.00	0-.65
	Sig.	0.21	0.18	0.74	0.49	0.68	0.37	0.52	0.82	.	0.07
Time of exposure	Cor Co	0.66**	0.02	0.23	0.05	0.14	-0.05	-0.05	-0.01	-0.26	1.00
	Sig.	0.0	0.91	0.05	0.66	0.26	0.69	0.68	0.94	0.07	.

**. Correlation is significant at the 0.01 level (2-tailed). *. Correlation is significant at the 0.05 level (2-tailed).
Cor Co = Correlation Coefficient, Sig. (2 tailed)

DISCUSSION

Gender determination is one of the first criteria for human identification. In forensic cases, whereby, the body is heavily fragmented and denatured, sex identification using DNA extracted from the teeth may be the only choice. In this study, the teeth were subjected to various environmental conditions: heated at 210^{o}C for 15 mins, buried in soil, immersed in Lagoon water, exposed to normal environmental conditions for 1–30 days, and to see the effects of these environmental conditions on DNA yield and sex determination.

This study used the dentine-cementum complex, as a source of DNA as previous studies have used this for identification purposes (Malaver and Yunis, 2003; Higgins *et al.,* 2011). No pulp was recovered in all the teeth. Factors that could be responsible for this could be, because the teeth collected for this study were removed based on therapeutic reasons e.g. periodontal disease, (Ibrahim *et al.*, 2016), previous endodontic therapy, (Higgins and Austin, 2013), hence a decrease in cell number and an increase in dentine sclerosis (Higgins *et al.,* 2013).

DNA extraction in teeth is affected by dental disease. With subsampling the dentin, cementum or both, DNA can be isolated (Higgins and Austin, 2013). In a study carried out by Higgins *et al.,* (2011), there was no significant difference in DNA yield between the dentine and cementum, and no significant difference between cementum in diseased and healthy teeth. Also, in a study carried out by Zapico and Uberlaker, (2013), DNA concentration values obtained from pulp and dentin were similar.

In this study, the mean DNA yield using the Silica column extraction method was high compared to the organic extraction method. In a study by Higgins *et al.,* (2013), nuclear DNA yield from cementum varied widely between teeth 0.28-173.57 ng/mg, using silica spin columns and qPCR. This was similar with our silica column extraction DNA concentration values.

There was a significant difference and positive correlation (at 0.05 level: 2 tailed) between organic method of extraction and silica column method. This was expected because the silica column method yielded more DNA, and this was similar to studies carried out by Pagan *et al.,* (2012). In a study by Raimann *et al.,* (2012), the use of isopropanol (organic method of extraction), gave a lower DNA yield, but contrarily a higher purity. However, in this study, DNA purity at 260/280nm was 0.97±0.07 using silica extraction and 0.91±0.11 using organic extraction. Though slightly increased, a higher purity was achieved with the silica column extraction method. This is contradictory to the report of Zapico and Ubelaker (2013), that achieved a higher DNA purity of 1.66 using the silica column method, without environmental exposure. This suggests that environment and length of exposure hypothetically affects DNA purity. Higher purity with the organic methods was achieved in studies by Raimann *et al.,* (2012), and Kumar and Aswath (2016). The low DNA purity, in this study, maybe due to variation between the individuals and teeth sampled, environmental factors and length of sampling, and also because crushed teeth may tend to introduce contamination, release bacterial endonucleases and PCR inhibitors. The effect of the environment of exposure on DNA concentration in this study showed a significant difference compared to the different groups. Even in the same environment, a wide variation in the concentration and purity of DNA has been reported (Muruganandhan and Sivakumar, 2011).

Teeth samples immersed in Lagoon water and buried in the soil gave the lowest DNA concentration in organic extraction methods and this was seen in similar studies (Musse *et al*., 2009; Pawar and More, 2018; Samsuwan *et al.,* 2018). This is due to autolysis and postmortem degradation that is enhanced by moisture, bacteria, humid bacterial present in this medium of exposure (Latham and Miller, 2018). In a study by Vermuri *et al.,* (2012), though DNA was extracted from pulp, samples that were immersed in lagoon for more number of days yielded less quantity of DNA, as DNA concentration values dropped from 34µg/ml on day 20 to 25µg/ml on day 28 when the chelex method of extraction was used. These values were lower than values from this study at 152.83±142.86ng/µl for organic extraction and 203.17ng/µl for silica column extraction for teeth immersed in lagoon for 21-30days. This is probably because of the difference in DNA extraction methods and type of sampling used.

Teeth exposed to normal environment and temperature of 210^{o}C for 15mins gave the highest concentration of DNA in the silica column and organic extraction methods respectively. However, the teeth exposed to normal environment for 21–30 days yielded no DNA after three trials using the organic method while DNA was extracted using the silica column method. This is probably because these samples were exposed to various environmental conditions e.g. high humidity, sunlight light, rain and dryness. Also, the use of silica method may improve DNA recovery in highly degraded samples. DNA Purity for organic methods of DNA extraction was highest for teeth exposed to temperature at 210^{o}C and were lowest in teeth exposed to Lagoon water for the two extraction methods. This was found in similar studies by Musse *et al.,* (2009), implying that water interfered in DNA preservation, with components of Lagoon water inhibiting PCR, such as sodium, calcium, chloride, magnesium, sulfate, potassium, and nitrate, trace elements (Alaeddini, 2012; Samsuwan *et al.,* 2018). In the organic method, teeth exposed to temp at 210^{o}C for 15 mins had DNA purity of 1.15±0.07. This is similar to the DNA purity (1.15 ± 0.05) obtained in studies by Mahat, *et al.,* (2019) using canine teeth exposed to heat at 300^{o}C for 20 min.

In this study, as the time of exposure increased, the mean DNA concentration decreased though the teeth used were exposed to different environments. DNA concentration was significantly correlated with the duration of exposure. This is similar to the study carried out by Khare *et al.,* (2018). In studies by Vermuri *et al*. (2012), the amount of DNA kept decreasing as the number of days increased.

There was also significant difference and correlation between DNA concentration and purity in both DNA extraction methods. Compared to DNA purity, time of environmental exposure within groups of environmental conditions, showed no significant difference. This was supported by a study carried out by Khare *et al.,* (2018) that showed that DNA purity was not significantly affected by the storage period of teeth in soil.

More premolars and molars were analyzed compared to the number of canine and incisors for this study. This is probably due to poor oral health because of their location so these types of teeth are more prone to disease as individuals age. The premolars and molars (upper and lower) gave the highest DNA concentration, while canine had the lowest. This is probably because molars and premolars are multi-rooted, hence having a large surface area of the root, more cellular cementum, compared with canines and incisors (single-rooted tooth) (Higgins and Austin, 2013). Raimann, *et al.* (2012), in their study, suggested that molars and premolars were good candidates to obtain DNA profiles irrespective of the type of the laboratory procedure used or the time the recovered body was decomposed.

Studies by Pawar and More (2018), showed no significant difference in DNA concentration between males and females, similar to this study however, DNA concentration was predominantly higher in male than females in both methods of extraction, together with DNA purity. Also, PCR identification of sex also showed far higher DNA concentration in females than in males, this is due to the negative identification of males in this study. In this study, Female positive identification was 100% while positive and negative male identification was 30.8% and 69.2% respectively. In a study by Zagga *et al.,* (2013a), there was a sensitivity of 33% when *amelogenin* gene was used as a marker to study sex determination in burnt skeletal fragments. In a study by Nayar *et al.,* (2017), they stated that sex determination was done with PCR method which showed 100% accuracy in Group I, 86% accuracy in Group II, 73% in Group III, and 86% in Group IV while Pawar and More, (2018) reported that the accuracy in determining sex from pulp DNA ranged from 92% to 100% in the study groups, except from the teeth exposed to uncontrolled heat, as the pulp tissue was burnt completely. In North western Nigeria, Zagga *et al*., (2013b) reported 100% *amelogenin* failure using dried human teeth, whereas Dutta *et al*., (2017) reported 100% retrieval of DNA along with gender determination successfully carried out in a short span of time with optimal technique sensitivity. *Amelogenin* sex test failures involve failure of AMELY to amplify, which results in the incorrect identifying of DNA samples from phenotypic males as female. DNA extracted from poorly preserved samples may yield spurious results as a result of allelic dropouts leading to misidentification of heterozygotes (XY) as homozygotes (XX), (Alvarez-Sandoval *et al*., 2014; Masuyama *et al*., 2017). DNA fragmentation occurs as a result of depurination and depyrimidination, and deamination which causes release of nitrogenous bases A, G, T, C, and an amino group, causing a baseless site hence making the DNA unstable (Masuyama *et al.*, 2017). Also, many *amelogenin* test failures have been reported to be due to large scale deletion events on AMELY and a theorized AMELY primer binding site mutation (Butler and Li, 2014). Problems like deletion of the Y copy of the *amelogenin* gene or AMELY-null and AMEL X null has been reported Alvarez-Sandoval *et al.,* 2014) and this study tends to support that. The use of *Amelogenin* gene as a marker for gender determination, has been termed unreliable and controversial (Zagga *et al*., 2013b; Laverde, 2013; Butler and Li, 2014). With problems like deletion of the Y copy of the *amelogenin* gene or AMELY-null and AMEL X null, no sequencing data are available yet for these samples, and even when alternate primers were used, there was no successful amplification of an AMELY null sample (Alvarez-Sandoval *et al.,* 2014; Butler and Li, 2014).

This study suggests that for teeth samples buried on sandy soil, immersed in lagoon or exposed to natural environment, silica column extraction method extracted more DNA compared to the organic extraction method, but for teeth samples heated at 210^{o}C for 15mins, organic extraction method yielded more DNA compared to silica column extraction method.

CONCLUSION

Within the limits of this research, freshly extracted teeth samples should have been used as controls and results should be treated as being applicable to only these environments of exposure, in context. Positive male and female identification were 30.8% and 100% respectively. Using the Nigerian population, further studies should be performed to ascertain the prevalence rates of AMEL Y null mutations, DNA degradation, large deletion events and or primer mutations as results from this work may well indicate higher prevalence.

Sex determination using *amelogenin* gene as a marker for gender determination, should be used together with other genetic gender determination marker(s) for increased specificity and sensitivity. No study to the best of our knowledge has attempted to compare simultaneously the effects of these environmental conditions on dentine cementum complex sample of the tooth and determine their effects on DNA concentration using the two techniques and also ascertain the sex of the individuals using *amelogenin* in Nigeria. In Nigeria, further studies to complement the findings of this study should be performed to ascertain the most reliable sex determination method in forensic science.

Conflict of Interest: None

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